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CANINE DETECTION OF ILLICIT DRUGS: SENSORY APPARATUS TECHNOLOGY

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13. ABSTRACT (Maximum 200 words)

This report describes basic anatomical and physiological observations of the peripheral canine olfactory system, provided by means of investigations into its structural and molecular characteristics. Canine olfactory receptor neuron electrophysiology was recorded intracellularly from epithelium strips. Intracellular recordings showed G-inhibiting protein to play a significant role to odorant response. In addition, RGS proteins which regulate the action of G-proteins were described for the first time in rodent and canine olfactory epithelium. We also show RGS to play a potentially important role in modulating olfactory neuron response to odorants. A complete three-dimensional nasal cavity was created from serially obtained CT scans. Volumes of respiratory, olfactory and sinus regions were determined from healthy intact animals. A 1:1 ratio between respiratory and olfactory region was observed. Modeling of air passage, airflow and distribution during sniffing events will now be possible. These results have provided the necessary background in understanding canine olfaction and allow further studies that could improve trace detection in dogs and aid in the development of trace technology.

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EXECUTIVE SUMMARY

The domestic dog remains as the most widely used fast, mobile and relatively accurate detection system available for locating odorous substances. Studies of canine olfaction are important for they are used by humans in detecting missing victims, uncovering drugs, currency and explosives. Despite dogs' legendary accomplishments in detection, little is known about the canine olfactory system. This project used a multifaceted approach which begins to unravel the complex structure and function of canine olfaction.

The canine olfactory epithelium consists of three cell types: supporting, receptor neurons and basal cells. The olfactory receptor neuron is bipolar. A dendrite extends to the epithelial surface where (~10-20) sensory cilia extend over the epithelium. A single axon transmits odorant information directly to an olfactory bulb. There was an extensive cell-cell connection throughout the full epithelial height between supporting cells and receptor neurons.

Canine olfactory receptor neuron electrophysiology was recorded intracellularly from epithelium strips. Intracellular recordings showed G-inhibiting protein to play a significant role in odorant response. In addition, RGS proteins, which regulate the action of G-proteins, were described for the first time in rodent and canine olfactory epithelium. We also show RGS to play a potentially important role in modulating olfactory neuron response to odorants.

A complete three-dimensional nasal cavity was created from serially obtained CT scans. Volumes of respiratory, olfactory and sinus regions were determined from healthy intact animals. A 1:1 ratio between respiratory and olfactory region was observed. Modeling of air passage, airflow and distribution during sniffing events will now be possible.

The project has provided basic anatomical and physiological observations of the peripheral canine olfactory system. Our results provide insights into the structural and molecular characteristics of the canine olfaction system. These results have provided the necessary

background in understanding canine olfaction and allow further studies that could improve trace detection in dogs and aid in the development of trace detection technology.

1.0 PURPOSE

The canine olfactory system has the remarkable capacity to detect and discriminate odorants from a myriad of odorant molecules. The task begins with olfactory receptor neurons, which convert information contained in the inspired air (odorant molecules) into membrane potentials. How the canine olfactory system completes these functions is not completely understood. The **goal** of this project was to examine the peripheral canine olfactory system. Planned experiments described and defined cytochemical, molecular, and physiological characteristics of canine olfaction. In addition, a three-dimensional model of the complex canine nasal cavity has been developed. Our results have provided insight into canine olfactory structure and function and could lead to improved substance detection and performance as well as the development of new technologies.

2.0 BACKGROUND

Humans rely preferentially on vision and audition as sensory complexes to keep us aware of our surrounding environment. However, our sensory legacy is not in our ability to see or hear but our ability to smell and taste. As a group, animals rely chiefly on their chemical senses, olfaction and taste. Olfaction is an ancient sense and one of the most important long-range sensory systems used in the detection of prey, predators, social interaction and reproduction. In mammals, the olfactory system can detect and distinguish a vast number of volatile substances that possess a variety of structures. Olfactory detection is mediated by the sensory epithelium which contains olfactory receptor neurons sensitive to various odorants and resides in a restricted portion of the nasal cavity. In mammals, the sensory epithelium covers a portion of the nasal septum and the thin, bony scroll-like ethmoturbinates and consists primarily of three cell types: non-sensory supporting cells, olfactory receptor neurons and basal cells (Morrison and Costanzo, 1990, 1992). Olfactory receptor neurons are bipolar cells having a dendrite that extends to the epithelial surface where it terminates in a knob-like swelling, from which arises a variable number of sensory cilia. The sensory cilia extend over the epithelial surface increasing the surface area, and contain membrane receptors for binding odorant ligand molecules present in the inspired air. Thus, one of the first events that must occur in the detection of an odor is to bring the odorant stimuli into contact with the sensory neuron, then bind the odorant to membrane receptors, thus exciting primary olfactory neurons. Inspired odorants activate membrane receptors located on specialized cilia and initiate the transduction of chemical stimuli into electrical signals. These signals are then transmitted as action potentials to the olfactory bulb of the central nervous system. Therefore, the number of olfactory receptor neurons and their distribution within the nasal cavity may play an important role in olfactory sensitivity (e.g. detection and discrimination).

Recently, there has been increased interest in the canine olfactory system. Studies of the canine olfactory system are particularly important for dogs, known to be one of the best odorant detection systems, and dogs are utilized by humans in tracking, rescue, explosive and drug

detection missions. Canine drug detection offers the most powerful and cost effective programs available to law enforcement agencies. In addition, the effectiveness and sensitivity of canine detection has provided the dog as the "gold standard" by which other detectors shall be measured (DARPA). It is generally accepted that dogs are effective in detecting a broad range of substances, and dogs are considered superior to machine and technology in a number of ways. The canine olfactory system is faced with a particular problem, the high dimensionality and inherent unpredictability of the chemical world. Most natural odors encountered by a detector dog in everyday life are a rich mixture of many different volatiles. This means that from the outset, a detector dog is confronted with a great and often unpredictable diversity of compounds. Despite the legendary ability of dogs to detect odors, little is known about olfactory morphology, cytochemistry, distribution of sensory neurons within the nasal cavity, and basic olfactory receptor neuron physiology of canine receptor neurons. An understanding of the mechanism of odor discrimination is, therefore, crucial to understanding canine olfactory behavior, detection and processes. Such information is important not only for understanding and enhancing canine olfaction, but also may play a role for the further development of trace-sensing technology.

3.0 OBJECTIVES

In order to better understand canine olfactory detection, discrimination and behavior, a thorough understanding of receptor neuron cytoarchitecture and receptor neuron functional physiology is necessary. We set out utilizing a multidisciplinary approach that included anatomy, neurobiology, physiology and biophysics imaging to study the sense of olfaction in dogs. Our three basic objectives were:

1. Examine the fine structure and cytochemical characteristics of canine olfactory sensory epithelium and regions of the nasal cavity.

2. Determine the odorant response and molecular characteristics of canine olfactory receptor neurons.

3. Determine the three-dimensional morphology and establish parameters for modeling the air flow and odorant distribution within the canine nasal cavity.

4.0 OBJECTIVES / METHODS

4.1 Objective #1: Structural Characteristics of Canine Olfactory Epithelium

Auburn University IAUCC approved all procedures in accordance with NIH guidelines for animal use. Light microscopy (LM) was used to examine the extent, relative distribution, and thickness (60-100 μm) of the olfactory sensory sheet within the canine nasal cavity. Scanning electron microscopy (SEM) determined the three dimensional structure of the sensory portion of the epithelium. SEM was particularly useful in that it allowed us to examine a large surface area and determine the cell surface characteristics which will help define sensory and non-sensory areas of the canine nasal cavity. SEM methods allowed us to examine fractured canine olfactory epithelium and determine the three-dimensional relationships among the cells of the sensory epithelium (Morrison and Costanzo, 1989). The importance of this procedure can be seen from our preliminary observations of canine olfactory epithelium (Morrison et al, 1999). Note the presence of numerous and extensive intercellular connections between supporting cells and olfactory neurons throughout the full extent of the epithelium (Figure 1). The importance of these cell-cell communications is unknown; however, they have proven to be a valuable observation related to our electrophysiological studies (see below). For example, in single cell dissociation preparations, the experiment may not yield reasonable results relative to in situ response of odorant (signature) samples. We developed a technique by which in situ examination of mammalian olfactory sensory epithelium can be achieved (see below).

In dogs, information concerning odor detection and discrimination in the environment are projected from the canine nasal cavity to circular patches of nervous tissue in the olfactory bulb called glomeruli (Figure 2). The olfactory glomerulus, one of the clearest anatomical modules in the CNS, is a complex and well-defined neuropil that is the site of first synapse in the olfactory pathway (Pinching and Powell, 1971; Shepard and Greer, 1998). In mammals olfactory glomeruli are 50-200 μm in diameter, comparable to barrels of the

somatosensory cortex and orientation columns of the visual cortex. The numbers of glomeruli vary in species: rabbit 2,000; rat 4,500; mouse 1,000. In dog, glomeruli ranged in size 100-200 μm and ~5,000 in number. The ultrastructure organization showed electron dense olfactory axons and electron lucent dendrites where synaptic profiles are present. Increasing evidence indicates that the olfactory glomerulus serves a fundamental unit for odor presentation. Recent studies have suggested that the intrinsic organization of single glomeruli although appearing homogenous, can be a heterogenous complex structure (Kasowski et al, 1999). Odor discrimination, recognition, and behavior patterns arise from the interactions of primary axons entering the olfactory bulb at the glomerulus. The interaction between glomeruli and the convergence ratio of receptor axons is not clearly understood in the dog and offers a rich area for further research. Further studies are needed to determine the synaptic organization of olfactory bulb glomerular compartments in canines. These studies in conjunction with receptor neuron projections could explain why canines are excellent detectors!

4.2 Objective #2: Odorant Detection and Molecular Characteristics

Following examination of the nasal cavity with combined morphological procedures, we were able to identify specific olfactory and non-sensory areas. Up to this point, most physiological studies were concerned with single cell response. We developed a method by which patches of olfactory epithelium (~5-30 mm^2) could be removed, positioned within a Ussing sample chamber, partially submerged, and remain viable for several hours (Figure 3). Our preliminary data obtained from rodent and canine olfactory epithelium have shown these experimental preparations of large olfactory mucosa sheets are physiologically viable for 6 to 12 hours. Interestingly, canine tissue typically remained robust for up to 24 hours. This method of study has several distinct advantages. SEM data showed the presence of extensive intercellular connections. These suggest that leaving tissue intact (as in our physiological preparation) would ensure that the olfactory epithelium suffers a minimal amount of damage, unlike single cell dissociation preparations. Olfactory epithelium

preparations that are completely saline-submerged typically survive for less than one hour. By removing the olfactory mucosa intact and attached to the underlying bone/cartilage and only partially submerging in saline, it became possible to obtain receptor neuron responses to odorants delivered in gaseous phase for up to 24 hours. This method has now made it possible to study regions of olfactory epithelium from specific identified areas. Future studies will allow us to (from specific signature odorants) directly record physiological responses and then do the cytochemical assays to correlate gene expression with physiological responses.

Our method of investigation offers the distinct advantage of simulating a normal canine nasal cavity environment. For example, the earliest single-cell recordings from vertebrate olfactory epithelium showed that individual olfactory receptor neurons (ORNs) typically respond to a range of odor substances which varied from cell to cell, but there was no way to determine cell location (Gesteland et al, 1965). It was suggested from these studies and also with insect studies that neurons could be divided into generalists and specialists. Whether excitatory or inhibitory, an ORN's response defines the range of odors that can elicit a response in a given cell. This has been referred to as the molecular receptive range (MRR) and is analogous to the receptive field in the visual system. Our system will allow us to record from specific areas and then determine the immunohistochemical and receptor profile.

At this time, we were now in a position to expose the canine olfactory epithelium to "micro puffs" of signature odorants (i.e. explosives, drugs) and record electrical physiological responses to different odorants for an extended period of time. This technique offers an opportunity to explore for the first time the molecular biology of canine receptor neurons within an intact epithelial sheet. Preliminary experiments have shown that canine olfactory tissue can be successfully removed enblock from the nasal cavity, "micro puff" stimulated, and carefully studied. Recording data showed the receptor neuron field to be a mosaic of excitatory and inhibitory type responses. Our results agree with the previous evidence that

the binding of odorant to an olfactory receptor protein leads to the activation of a specific stimulatory G-protein which amplifies the reaction of the receptor. The G-protein gives the information to an enzyme adenylcyclase second messenger. As a result, in the presence of ATP the cell can produce cAMP in large quantities. Therefore, by the activation of only one receptor protein by an odorant molecule, many cAMP molecules can emerge, each one capable of opening ionic channels (Sinnarajah et al, 1999; Zufall et al, 1993). As a result, the membrane depolarizes. Our laboratory recently showed not only the involvement of *Golf* but also Gi, an inhibitory G-protein (Figure 4; Sinnarajah et al, 1998) in response to odorant stimulation. In addition we reported for the first time the presence (Figure 2) and involvement of regulator of G-proteins (RGS) in olfactory neuron transduction (Sinnarajah et al, 1999, Dennis et al, 2000) (see below). Our experiments showed RGS proteins to be present in canine olfactory tissue and, specifically, RGS2 decreases cAMP accumulation, a significant finding. This important area of study is still progressing.

One unique feature of the olfactory system is the ability to adapt after being exposed to a continuous odor. Likewise, another important feature is the olfactory system's ability to rapidly recover, which would be extremely important in tracking a specific odor signature (e.g. prey, drugs, explosives). In addition, canine detectors are confronted with a number of odorants during their detection trials. Previous studies indicate the most common result of mixing two or more odors is masking (decreased perception) of one or both components. The initial response of an ORN to an odor stimulus is typically followed by a period of reduced responsiveness. This is evident both in a decline of the response to a maintained stimulus and in reduced responses to repeated stimuli. This reduction in responsiveness is termed sensory adaptation, simply the ability to "adapt" to an odor by not apparently detecting. Several mechanisms can contribute to adaptation. Intracellular Ca^{+}, intracellular kinases, and carbon monoxide at low levels have all been postulated to play a role in olfactory adaptation function. Our preliminary experiments indicate that RGS proteins may play a role in olfactory adaptation (Figure 5). The mechanisms underlying adaptation and masking are largely unknown. Our laboratory is positioned to explore the cellular

mechanism that may explain how receptor neurons function during direct stimulation and masking events, by examining second messenger responses. For example, G-proteins play an important role in excitation and inhibition of olfactory receptor neurons. In addition, the involvement of RGS proteins is just beginning to be explored. It is believed that RGS family may play an integral role in fine-tuning the second messenger of olfactory neuron response. It is expected that these results will provide new insights into olfactory neuron response to single and multiple odor stimuli, adaptation, and the potential masking of an odorant stimuli.

4.3 Objective #3: Three-dimensional Reconstruction and Modeling of the Nasal Cavity

In the canine nose, odorant molecules do not have immediate access to receptor neurons. Inspired air must traverse a complex array of thin wafer-like bony turbinates. This complex bony formation is covered by an epithelium that functions to warm, clean and humidify inspired air before it reaches the sensory region. Odorant molecules are then transported to the surface of the mucus covering the underlying sensory cilia.

An aspect of canine detector dogs rarely considered is sniffing behavior and associated with this, the manner in which odor molecules enter and are distributed within the nasal cavity. Among mammals (in particular dog), sniffing is a conspicuous behavior but little studied. A preliminary study was performed by IBDS using dogs trained to wear a thermocouple attached to their muzzle at the entrance to the left or right nares (CBD#10A). The basic phenomenon of sniffing behavior was described. Both short and long sniff patterns have been observed. We examined the distribution of fine (0.5 - 5 μm particle size) charcoal powder following active sniffing episodes in five dogs. Charcoal was predominantly found in the anterior (ventral) nasal cavity ventral to the maxiloturbinate. When an animal was involved with vigorous active sniffing, particles reached as far as the ethmoturbinates. These preliminary examples illustrate the complexity of air flow in the *in situ* nasal cavity. During sniffing, air flow which normally takes a rather direct route to the pharynx is transiently increased and eddies drawn over and

around the complex turbinates (Figure 6). This airflow may increase exposure to the receptor field and also turbulence helping to mix and distribute odorized air (Mozell et al, 1991). A major obstacle present in studying detailed airflow patterns of the nasal cavity is the extremely complicated nasal cavity anatomy. In order to address this problem we explored the usefulness of obtaining CT images from healthy canine non-terminal animals. Coronal CT scans were serially taken 5 mm, from the tip of the nares to the cribriform plate. The outlines of the cross sections were rendered and compiled to form a three-dimensional reconstruction of the canine nasal cavity (Figures 6b and 6c). By correlating CT images with known histological slides and gross anatomy we were able for the first time to calculate volumes of respiratory air (41.2 cc), sinus air (85.5 cc) and olfactory air (55.9 cc) derived from non invasive imaging (Morrison et al, 1999). It is expected that these results will provide a three dimensional analysis of the nasal cavity to which it will then be possible to apply computational fluid dynamic software (Fluent Inc.) to calculate air flow through the canine nasal cavity (Figure 7). We now feel we are in a position to determine laminar flow patterns of odors inspired during sniff episodes (short and long duration). The results of this study should provide, for the first time, insight into where specifically odorant molecules enter and distribute within the complex canine nasal cavity, potential storing of odorant air, washout, and air flow dynamics. The information obtained from this study will not only provide insight into the function of canine olfaction (and other vertebrates notably humans), but also aid in the further development of trace detectors.

5.0 SUMMARY

Understanding the canine olfactory system is important for several reasons. The use of canines in a variety of detection events is based on a dog's ability to detect and discriminate a specific odor among a myriad of odorant components. How a detector dog accomplishes this remarkable feat is not well understood, but the canine detector dog and handler are still considered the best deterrent in the field. The results of our project have provided for the first time a careful examination of structure and function of the canine olfactory system. We have made significant findings in the basic understanding of the structure of the functional unit of odorant detection, the olfactory sensory neuron, the physiological molecular response to different odorants, and developed a three-dimensional model system to determine flow patterns of inspired air. Our results have provided the necessary background in understanding the remarkable ability of canine detectors and a better understanding of detection and discrimination. Our research has resulted in four published abstracts, one proceeding contribution, one Gordon Conference presentation, one manuscript in reviewed (Nature, 2000), and one manuscript in preparation (Chemical Senses).

6.0 EXPLANATION OF FIGURES

Figure 1. **A.** Single dendrite (d) pulled away from epithelium shows the dendritic knob and sensory cilia (arrows). **B.** Sensory surface of canine olfactory epithelium with dendritic knobs and sensory cilia (arrows) among microvilli of supporting cells. **C and D.** Scanning electron micrograph of a fractured canine olfactory epithelium illustrating three-dimensional structural relationship. Arrow heads show extensive intercellular connections between supporting (s) and olfactory receptor neuron cells (o) within the epithelium.

Figure 2. Low power light micrograph illustrating nasal cavity (c) lined by sensory olfactory epithelium, olfactory bulb (O) and cerebrum (Cb). Around the periphery of the olfactory bulb are located circular neuropil called glomeruli (arrows), where the first synapse occurs. **B.** Silver stain preparation showing dendrite process of mitral cell extending to glomeruli. **C.** Electron micrograph of glomerulus shows the electron dense olfactory receptor neuron axon processes and lucent mitral/tufted cell dendrites (d), synapse appositions (arrows).

Figure 3. This illustrates the apparatus developed for obtaining electro-physiological recordings from canine olfactory mucosa. This method is advantageous to single cell preparations in that it provides a "normal "*in situ*" environment. After obtaining physiological data we will be able to process tissue to correlate histochemical findings. **B.** Current response recordings showing normal and recordings from olfactory neurons within mucosa following microinjections of antibody into olfactory receptor neurons from canine olfactory mucosa tissue. Note the response following injection of anti Gi showing greater depolarization wave.

Figure 4. Olfactory epithelium processed for double labeled immunohistochemistry showing RGS (green) and NCAM (orange). RGS protein stained green (arrow heads) is localized specifically in olfactory epithelium. Neural cell adhesion molecule stained orange (arrow) is demonstrated within the tissue colocalized with ORN.

Figure 5. The complex three-dimensional anatomy of the canine olfactory epithelium is illustrated in 5A. Olfactory receptor neurons (o) and the extensive cell-cell connections (arrowheads) are present throughout the entire epithelium. Olfactory neurons are bipolar, having an apical dendrite (d) extending to the epithelial surface and a basal axon (arrow) process projecting to the brain. We isolated fragments of olfactory mucosa (see Figure 3) and pass a microelectrode through the epithelial surface into a receptor neuron. When an odorant mixture is puffed onto the surface (*) we then record the neuron response, such as demonstrated in Figure 5B, Trace A. When the microelectrode is loaded with RGS2/3 antisera, the odorant induced currents are dramatically changed (Figure 5B, Trace B).

Figure 6. **A.** Gross dissection of canine nasal cavity showing complex turbinate structure and large olfactory bulb (o) where olfactory axons project. Arrow head illustrates extent of charcoal deposition following sniff events. **B.** Shows the three dimensional reconstruction from 5 mm CT serial sections of respiratory (r), olfactory (o), sinus (s) nasal cavity. Nasopharyngeal (p) region dark green. Relative air volume determinations are: total volume 181.6 cc (respiratory: 41.2 cc, olfactory: 55.9 cc, sinus: 84.5 cc). **C.** CT reconstruction showing sensory olfactory area (o) that projects to the olfactory bulb of the central nervous system.

Figure 7. This is a preliminary three dimensional composite created from CT scans serially collected through the canine nasal cavity using FIDAP software. These reconstructions represent the anterior half of the complex nasal cavity. Nostril opening depicted at arrow. Arrowheads represent nasal air cavity of the maxiloturbinate region.

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FIGURES

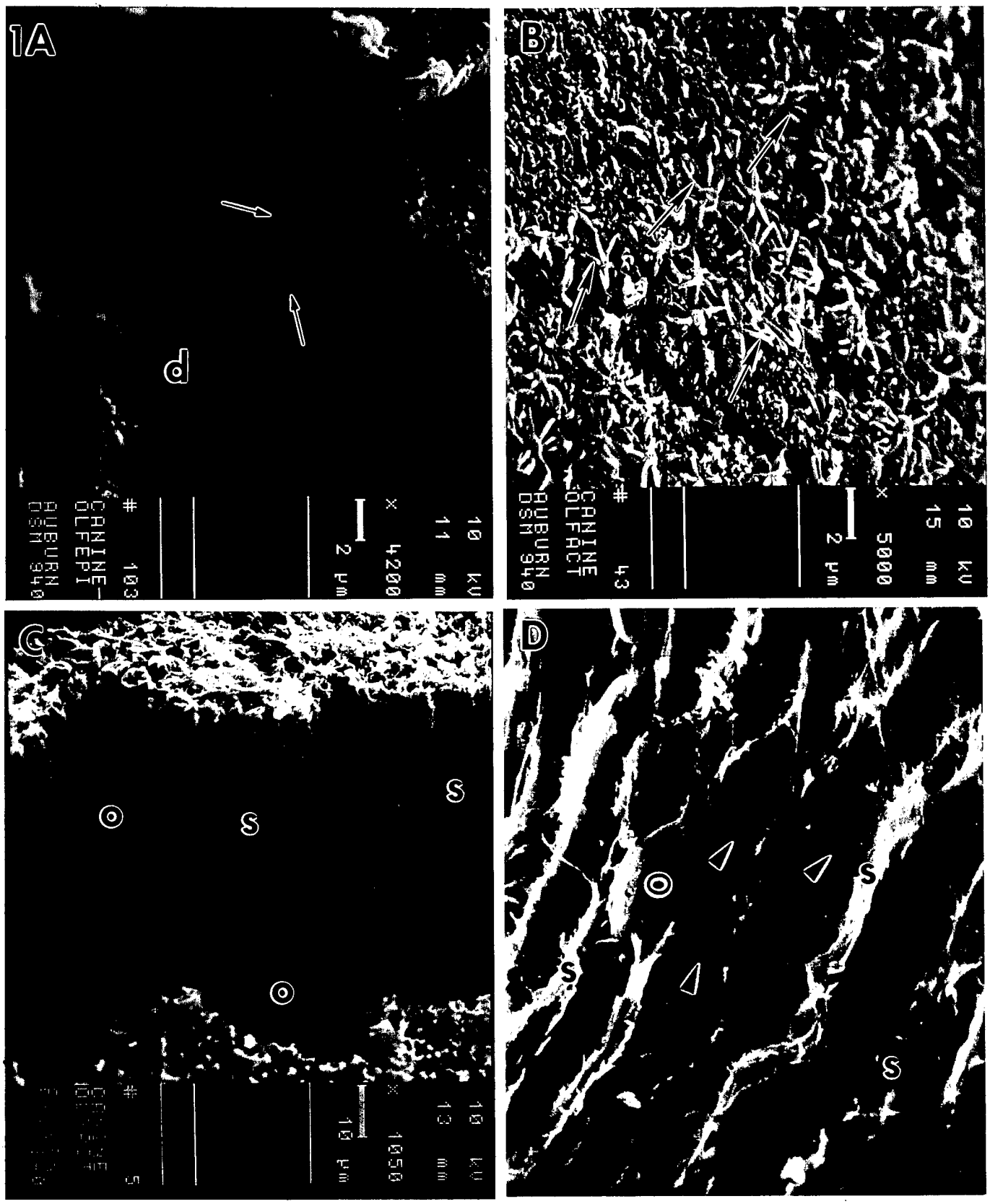
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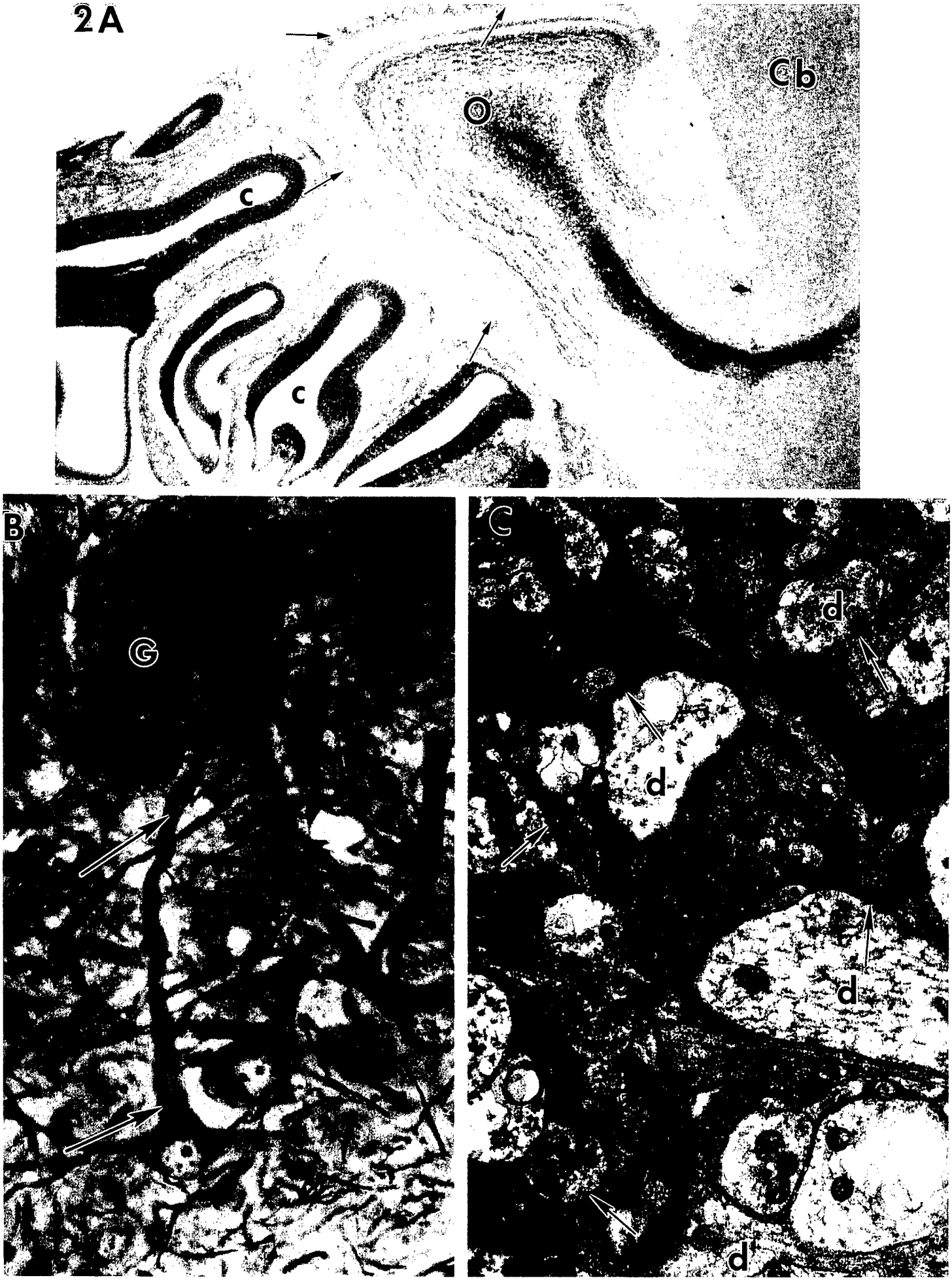
FIGURE 1



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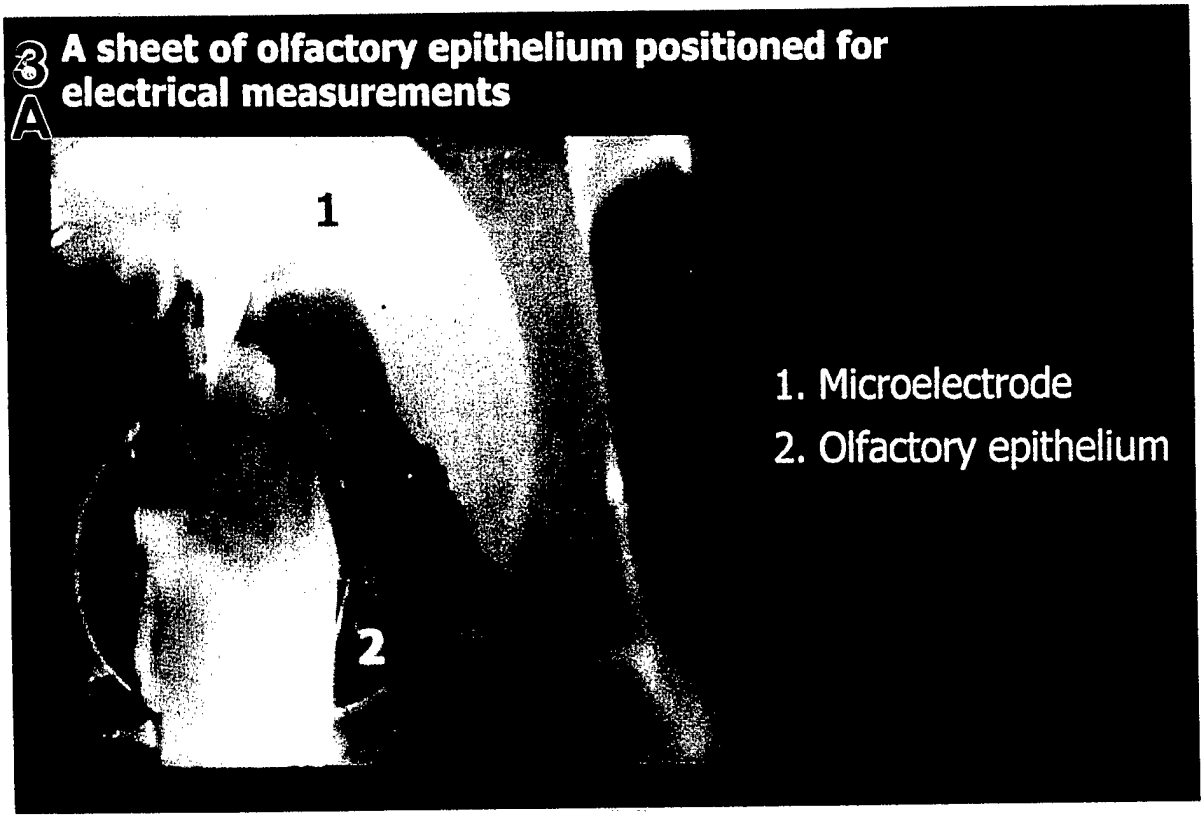
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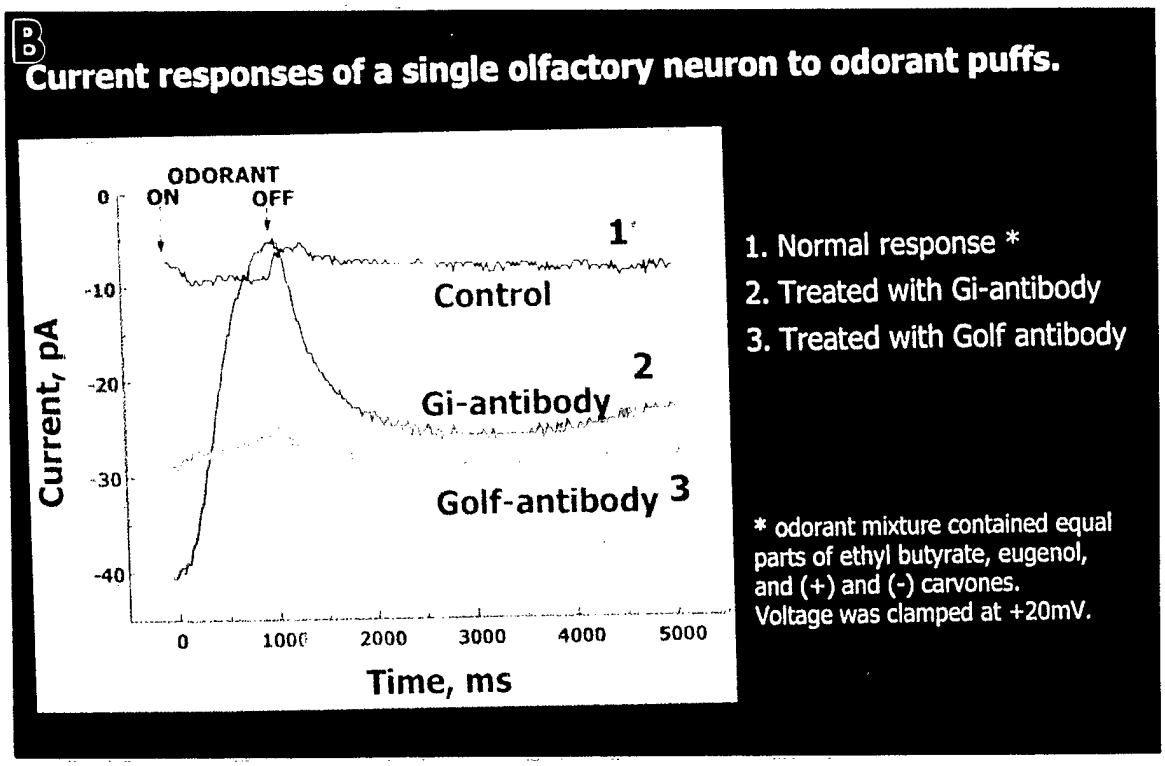
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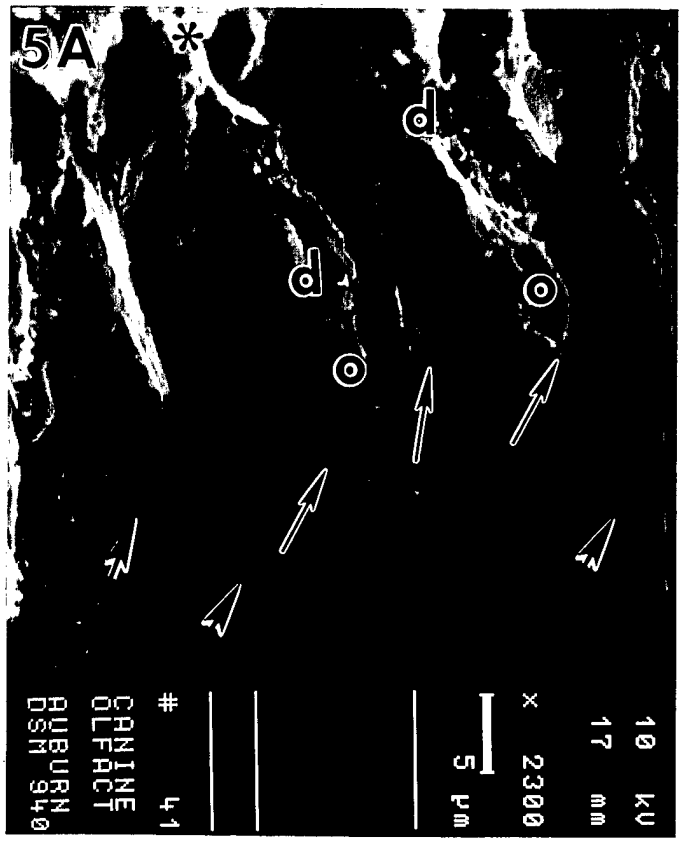
* odorant mixture contained equal parts of ethyl butyrate, eugenol, and (+) and (-) carvones. Voltage was clamped at +20mV.

FIGURE 3

FIGURE 4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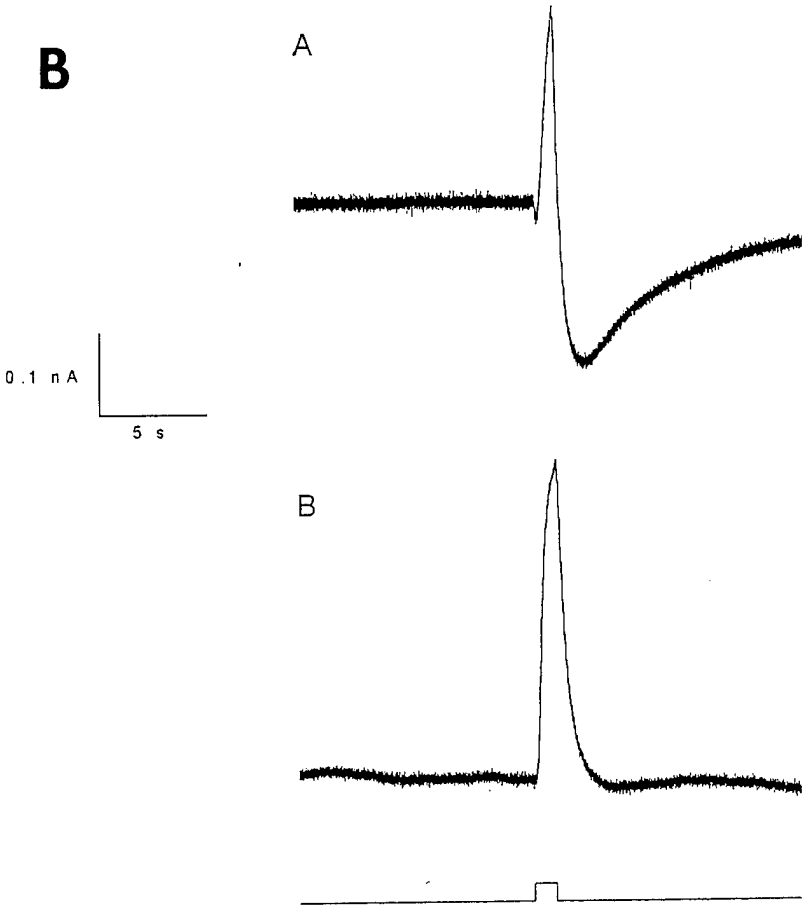
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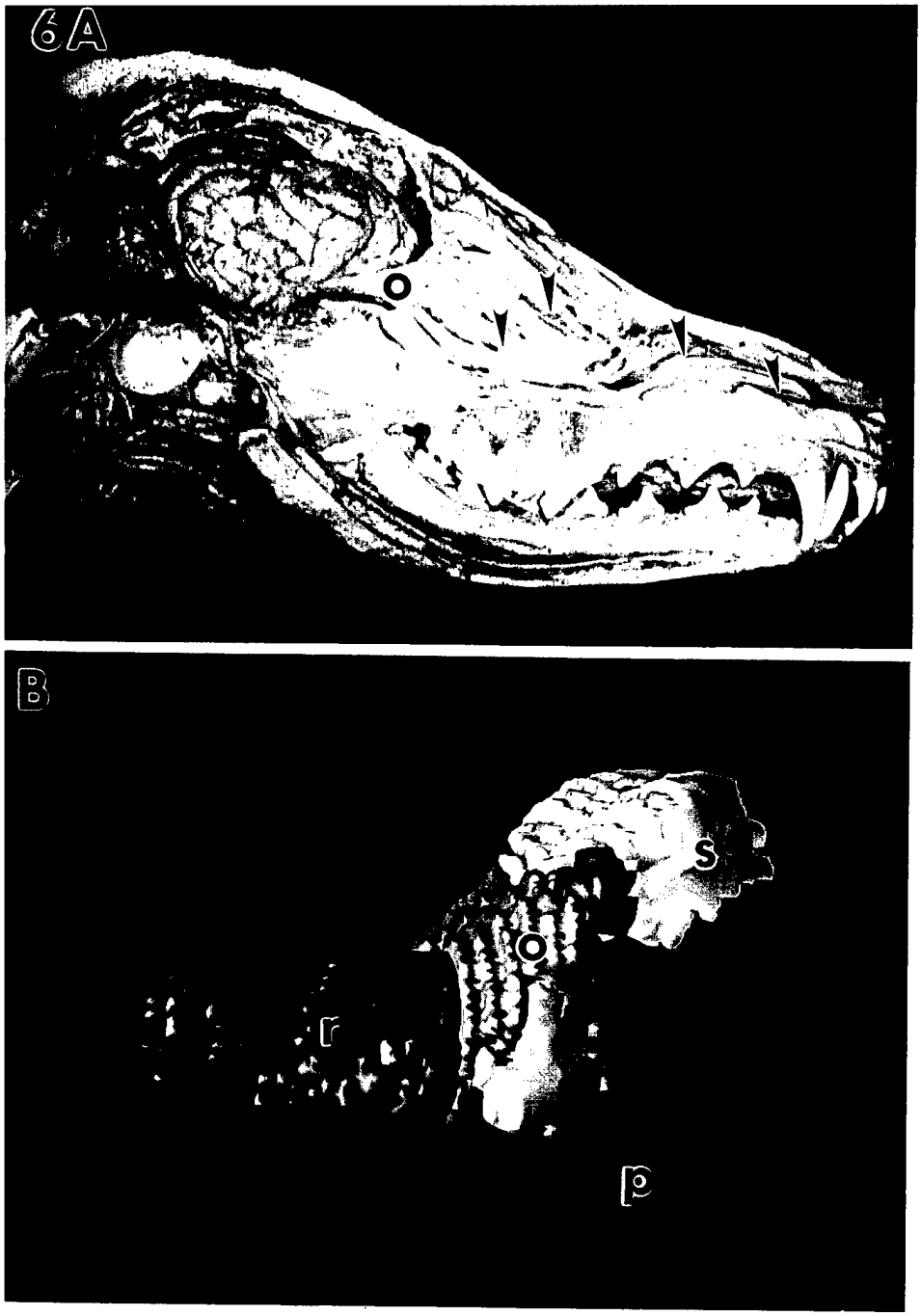

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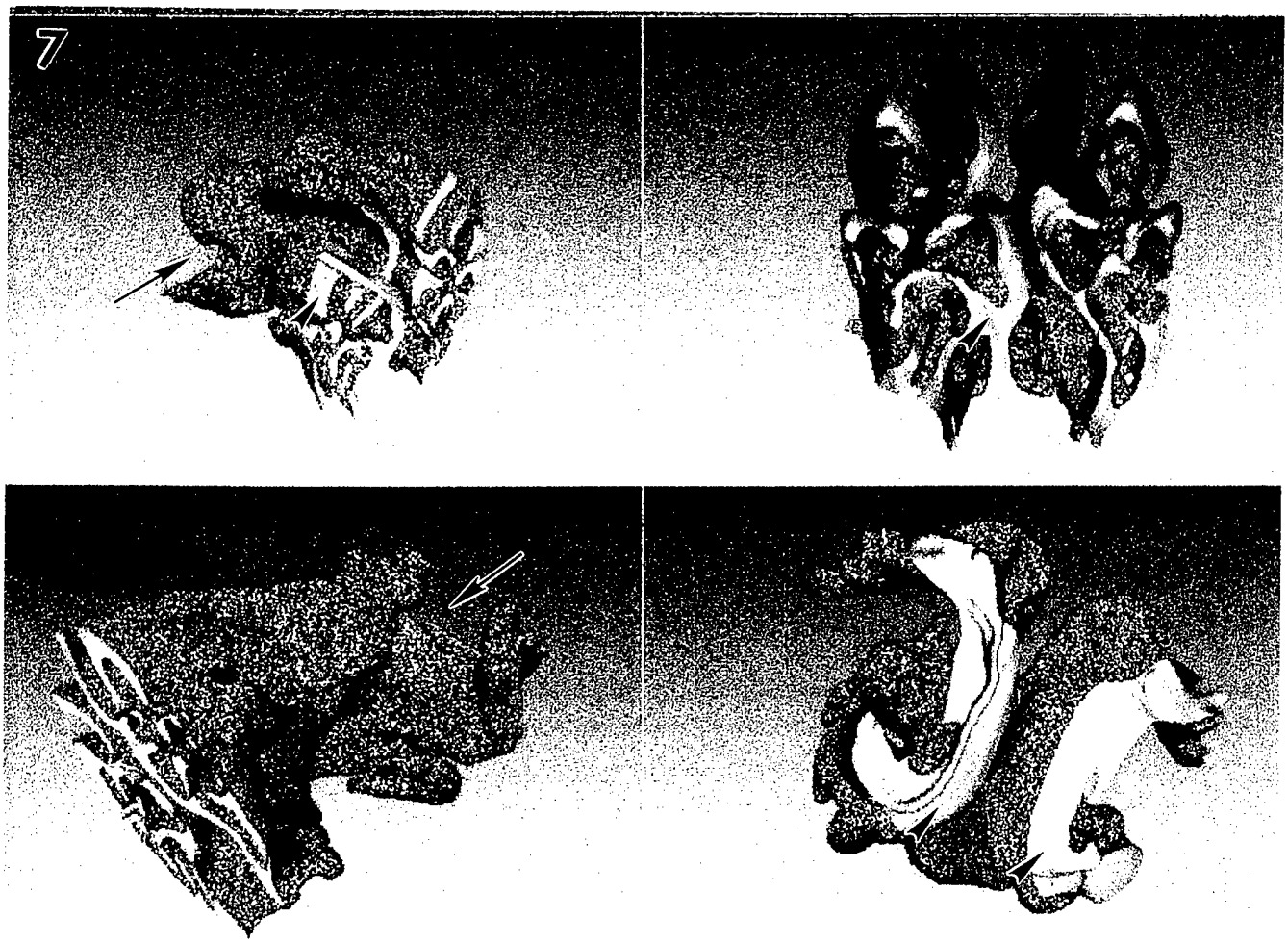

FIGURE 8

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